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Supercritical fractionation of rosemary extracts to improve antioxidant activity

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Abstract

A supercritical CO2 pilot plant was employed to extract rosemary (Rosmarinus officinalis L.) leaves and to thoroughly study the effect of time on the yield, composition and antioxidant activity of the different fractions produced. Six extractions assays were carried out varying the fractionation procedure using a two-stage depressurization system coupled to the extractor vessel.

The concentration of the main antioxidant compound in rosemary, namely carnosic acid, and the DPPH test were employed to value the antioxidant power of the different fractions obtained. The goal has been a new fractionation scheme, comprising two different periods of time, in which the exhaustion of the essential oil from plant matrix was achieved, while the recovery of the antioxidant compounds was maximized.

1. Introduction

Among the natural sources of bioactive compounds, many herbs have been applied in folk medicine since ancient times because they have been recognized to have medicinal properties (antiseptic, antifungal and antimicrobial). More recently it has become clear that another valuable property of many spices, for example rosemary, sage, thyme, clove, oregano, is that they contain natural antioxidants.

Antioxidant compounds in food play a very important role. Oxidation is one of the major causes of chemical spoilage, resulting in rancidity and/or deterioration of the nutritional quality, color, flavor, texture and safety of foods. Modern consumers ask for natural products, free of synthetic additives, and therefore several spices and some herbs have received increased attention as sources of effective natural antioxidants. But besides their role as food stabilizers, they can protect cells against the effects of free radicals and thus, play an important role in heart disease, cancer and other diseases [1].

Indeed, supercritical fluid technology is the most innovative method to recover bioactive compounds for use as supplements for functional foods. Extraction of plant material using supercritical CO_2 (SC-CO_2) has been the subject of several publications; the reader is referred to recent reviews presented in references [2-4]. Particularly, SC-CO_2 extraction to recover antioxidant compounds from different Lamiaceae herbs is being a matter of continuous research [5].

Rosemary (*Rosmarinus officinalis L.*) has been recognized as one of the Lamiaceae plants with large antioxidant activity. Main substances associated with the antioxidant activity are the phenolic diterpenes such as carnosol, rosmanol, carnosic acid, methyl carnosate, and phenolic acids such as the rosmarinic and caffeic acids [6-10]. Particularly, carnosic acid has been recognized as the most abundant antioxidant compound present in rosemary extracts.

Different authors [11, 12] compared supercritical rosemary extracts with those obtained using liquid solvents (ethanol and hexane) or hydrodistillation, and demonstrated the superior antioxidant activity of the supercritical extracts.

The SC-CO_2 extraction of rosemary leaves to produce natural antioxidant products has been widely studied and reported in the literature [11-22]. Furthermore, SC-CO_2 extraction is being employed by several companies to produce rosemary antioxidant extracts. Despite the commercial and scientific interest on the antioxidant compounds present in rosemary, small amount of work has been reported about the solubility of such substances in SC-CO_2 [23].

Definitely, the antioxidant power of the extracts produced depends not only on process conditions, but also on the origin of the raw material employed. For example, Carvalho [11] studied the supercritical extraction of organic cultivated rosemary (Sao Paulo, Brazil) in low-scale extraction cells of diverse size. Different extraction conditions were applied, and an extract containing around 20 % wt of carnosic acid was obtained at 30 MPa, 313 K, using pure CO_2 and without any fractionation of the extract. The overall extraction yield obtained was 0.05 g of extract per g of plant material loaded to the extraction cell (5 %wt).

In general, lower yields and concentrations of carnosic acid were obtained by other authors. Celiktas [15] extracted antioxidant fractions from rosemary leaves collected from different locations of Turkey, and at different harvesting time intervals: at 35 MPa, 373 K and with 5% of methanol as co-solvent, the carnosic acid content in the extracts varied from 0.5 to 11.6 % wt. The rosemary plants studied by Chang [16] were grown in experimental fields of Taiwan; they explore pressures in the range of 20-35 MPa and temperatures of 313-343 K; the best antioxidant extract was obtained at 35 MPa and 343 K, with 4.3% overall yield and carnosic acid content of 3.5 % wt. In previous work

[19], we extracted rosemary leaves from Murcia (Spain) in a pilot-scale plant at 30 MPa, 313 K and with pure CO_2, obtaining an overall extraction yield of 4.5 % and a carnosic acid content around 10 % wt.

Furthermore, fractionation of the extract has been proved to be an efficient procedure to concentrate the phenolic compounds in one fraction and thus, a product with improved antioxidant power can be obtained. In general, fractionation was accomplished by applying different conditions in two time sequential extractions (sequential fractionation) or by producing a cascade decompression of the extract in two or more separator vessels (on-line fractionation). For example, Ibañez [13] used sequential fractionation to isolate volatiles at 10 MPa and 313 K, while the antioxidant fraction was obtained at 40 MPa and 323 K without using a cosolvent. Ivanovic [20] employed similar sequential fractionation scheme: a first fraction comprising essential oils was extracted at a pressure of 11.5 MPa and 313 K, following by the antioxidant fraction which was extracted at 30 MPa and temperatures in the range 313-373 K.

On-line fractionation in a two-step depressurization system was studied by Cavero [14]: extractions were carried out at 15-35 MPa and 313-323 K, with 0-7 % of ethanol; the antioxidant fraction was isolate in the first separator (7.5-14 MPa), while the volatiles were recovered in a second separator (2 MPa). The best separation was achieved when the highest extraction pressures and no cosolvent were employed.

Despite the origin of rosemary leaves, the selected extraction conditions or the fractionation method applied, it has to be taken into account that the composition of the extracted material varies significantly with extraction time. Decreasing percentages of lighter compounds (terpenes and oxygenated terpenes) were found as extraction time increase, while higher-molecular-weight compounds (diterpenes and sesquiterpenes and oxygenated derivatives) showed a continuous percentage increase at increasing

extraction times [25]. Thus, on-line fractionation of rosemary extracts should also vary with time in order to produce a selective separation of the antioxidant substances and maximize their yield and concentration.

In previous work [19], we studied the kinetic behavior of rosemary supercritical extracts with respect to yield, antioxidant activity and carnosic acid content. Extractions were carried out without cosolvent, at 30 MPa and 313 K, and during 8 hours. A significantly increase of carnosic acid concentration was observed, from 7.8 % wt in the fraction collected during the first two hours to 28.0 % wt in the fraction collected during the last two hours. Consequently, the antioxidant activity of the fractions collected increased considerably with increasing extraction time.

Based in these results, a novel fractionation procedure was investigated in this work, in which two different fractionation schemes are combined, in order to maximize not only the antioxidant activity but also yield of the rosemary antioxidant fraction produced.

2. Materials and methods

2.1 Chemicals

2, 2- Diphenil-1-pycril hydrazyl hydrate (DPPH, 95% purity) were purchased from Sigma-Aldrich (Madrid, Spain). Carnosic acid (≥96%) was purchased from Alexis Biochemical (Madrid, Spain). Ethanol and phosphoric acid (85%) were HPLC grade from Panreac. Acetonitrile was HPLC grade from Lab Scan (Dublin, Ireland). CO_2 (N38) was supplied from Air Liquid.

2.2 Rosemary leaves preparation

The rosemary sample (*Rosmarinus officinalis L.*) consisted of dried rosemary leaves (water content < 5 % wt) obtained from an herbalist's producer (Murcia, Spain). The sample was ground in a cooled mill and sample was sieving to the appropriate size (between 200 and 600 μm).

2.3 Supercritical extraction and on-line fractionation procedure

Extractions were carried out using a pilot-plant supercritical fluid extractor (Thar Technology, Pittsburgh, PA, USA, model SF2000) comprising a 2 L cylinder extraction cell and two different separators (S1 and S2), each of 0.5 L capacity, with independent control of temperature (± 2 K) and pressure (± 0.1 MPa). Figure 1 presents a simplified scheme of the supercritical extractor employed. The extraction device also includes a recirculation system, were CO_2 is condensed and pumped up to the desired extraction pressure. For each experiment, the extraction vessel was packed with 0.55 kg of rosemary. Extraction assays were performed at 30 MPa and 313 K, with an upwards CO_2 flow rate of 60 g/min. Temperature was set to 313 K in both S1 and S2 separators. According to previous kinetic studies [19] the overall extraction time was set to 5 h.

Six SC-CO_2 extractions were carried out at the extraction conditions given above, but varying the on-line fractionation scheme, as reported in Table 1.

Extraction 1 was carried out with no fractionation of the extract, and thus all extracted material was recovered in the first separator (S1), which was maintained at the recirculation system pressure (5 MPa).

On-line fractionation of the extract was accomplished in Extractions 2 to 6, but the fractionation period (EP1 hours) was varied, starting with EP1=1 in Extraction 2, up to Extraction 6 in which fractionation was carried out during the whole extraction time (EP1=5). During the fractionation period, S1 was set to 10 MPa, while S2 was

maintained at the recirculation pressure. After period EP1, the pressure in S1 separator was lowered down to CO_2 recirculation pressure (5 MPa). Two different extracts were collected in separators S1 and S2. Ethanol was used to wash out the separators and ensure a complete recovery of the material precipitated in each cell. Ethanol was eliminated by evaporation and the homogeneous solid samples obtained were kept at -20°C under N_2 and in the dark until analysis.

2.4 GC-MS analysis

Essential oil content and profile of samples was determined using a GC-2010 (Shimadzu, Japan). The column employed was a ZB-5 (Zebron) capillary column, 30 m x 0.25 mm I.D. and 0.25 µm phase thickness. Helium, 99.996% was used as a carrier gas at a flow of 1 ml/min and inlet pressure of 57.5 KPa. Oven temperature programming was 60ºC isothermal for 4 min then increased to 106 ºC at 2.5 ºC/min and from 106ºC to 130ºC at 1ºC/min and finally from 130ºC to 250 ºC at 20ºC/min, this temperature was kept constant for 10.2 min. Sample injections (1 µl) were performed in split mode (1:20). Injector temperature was of 250ºC and MS ion source and interface temperatures were 230 and 280ºC, respectively. The mass spectrometer was used in TIC mode, and samples were scanned from 40 to 500 amu. The compounds were identified by comparison with mass spectra from Wiley 229 library. GC-MS analyses were carried out by duplicate and the average standard deviation obtained was ± 0.08%.

2.5 HPLC analysis

The carnosic acid content in the samples was determined using an HPLC (Varian Pro-star) equipped with a Microsorb-100 C_{18} column (Varian) of 25 cm × 4.6 mm and 5 µm particle size. The analysis is based on the work of Almela [25]. The mobile phase

consisted of acetonitrile (solvent A) and 0.1% of phosphoric acid in water (solvent B) applying the following gradient: 0–8 min, 23% A, 8-25 min, 75% A, 25-40 min 75% A and the 40-45 min 23% A . Initial conditions were gained in 5 min. The flow rate was constant at 0.7 ml/min. Injection volume was 20 µl and the detection was accomplished by using a diode array detection system (Varian) storing the signal at a wavelength of 230, 280 and 350 nm. Quantification of carnosic acid was performed by a calibration curve of the pure standard in all samples (y =2.62 10^8 X-1.05 10^6, R^2=0.998). Samples were analyzed by HPLC in duplicate and the obtained average standard deviation was ± 0.13%.

2.6 Antioxidant activity by the DPPH test

The method consists in the neutralization of free radicals of DPPH by an antioxidant sample [26]. An aliquot (50 µl) of ethanol solution containing 5-30 µg/ml of rosemary extract, was added to 1.950 µl of DPPH in ethanol (23.5 µg/ml) prepared daily. Reaction was completed after 3 h at room temperature and absorbance was measured at 517 nm in a Nanovette Du 730 UV spectrophotometer (Beckman Coulter, USA). The DPPH concentration in the reaction medium was calculated from a calibration curve determined by linear regression ($y = 0.0265 \cdot x$; $R^2 = 0.9998$). Ethanol was used to adjust zero and DPPH-ethanol solution as a reference sample. The amount of extract necessary to decrease the initial DPPH concentration by 50% or EC_{50} (µg/ml) was determined and employed to value the antioxidant power of the sample; the lower the EC_{50}, the higher the antioxidant power.

3. Results and discussion

As mentioned before, the extraction temperature and pressure were kept constant at 30 MPa and 313 K during Ext. 1 to 6 (see Table 1) but the depressurization procedure adopted to fractionate the material extracted was varied with respect to time.

Two different extraction periods were considered. During the first extraction period (EP1 hours) pressure was set to 10 MPa in the first separator (S1) while the second separator (S2) was maintained at the recirculation system pressure (5 MPa). Thus, during EP1, fractionation of the material extracted from the plant matrix is produced by an on-line cascade decompression. Due to the lower solubility of the antioxidant compounds (phenolic-type substances) in comparison to the essential oil compounds (mainly monoterpenes) it is apparent that the antioxidants would precipitate in S1, while the essential oil would be mainly be recovered in S2.

During the rest of the extraction (the second extraction period is EP2 = 5 - EP1) the extract is no further fractionated, S1 pressure is lowered down to CO_2 recirculation pressure, and thus, all the substances extracted were precipitated in S1 and mixed with the material that had been already recovered in S1 during EP1.

As shown in previous work [19], the antioxidant activity of the extracted material increase as extraction time increase. This could be attributed to the exhaustion of the volatile oil from plant matrix and the consequent concentration of phenolic-type compounds. Thus, the extent (hours) of EP1 was varied to determine the fractionation time required in order to remove the essential oil compounds from the rosemary leaves matrix. The extent of EP1 in the different supercritical extraction assays is indicated in Table 1.

Figure 2 show the extraction yield attained in S1 and S2 as a function of the fractionation time (EP1). The overall extraction yield obtained, i.e. considering S1 and S2 extracts, was 4.15 %, which is the mean value of Ext. 1 to 6 given in Table 1

(standard deviation = 0.18). With respect to extraction yield, as expected, the amount of material recovered in S1 decreases when EP1 increases, whilst the mass recovered in S2 increases. Thus, the lowest yield was obtained in S1 (i.e. the separator in which it is expected to recover the antioxidant compounds) when fractionation was accomplished during the whole extraction time (Ext. 6 on Table 1).

The results obtained from the analysis of the volatile oil fraction are given in Tables 2 and 3. According to Table 2, considering the % areas of the identified substances, the main components comprising rosemary essential oil are 1,8 cineole and camphor, followed by borneol and α-terpineol. Further, similar % area were obtained in most of the samples analyzed and thus, it could be stated that the volatile oil composition of fractions collected is rather similar (see Table 2). Nevertheless, it has to be pointed out that the ratio (total area identified in S2) / (total area identified in S1) is 1.6 (Ext. 2) to 6.2 (Ext. 6) signifying that, as EP1 increases, significantly lower amounts of volatile oil compounds are recovered in S1.

Table 3 gives, for each component *i*, the ratio r_i between its chromatographic area in the different fractions collected and its chromatographic area in fraction S1 of Ext. 1 (i.e. the experiment in which no fractionation was accomplished). Since all samples were injected at the same concentration in the GC-MS system, r_i is a relative measurement of the concentration of component *i* in the corresponding fraction. According to Table 3, the main constituents of rosemary essential oil are concentrated in S2 separator: a 2-3 fold increase is observed for several substances, such as 1,8 cineol, linalool, camphor, borneol, verbenone and β-caryophyllene. On the contrary, in S1 fractions the concentration factors (r_i) of the essential oils compounds are in general lower than 1.

The concentration of the main antioxidant compound detected in the HPLC analysis (carnosic acid) is reported in Table 4, together with the EC_{50} values resulted from the

DPPH analysis. Carnosol was also identified as an antioxidant compound present in the samples, but very low amounts of this substance was determined (< 2 %wt).

Considering the total amount of carnosic acid (CA) extracted in Ext. 1 to 6 of Table 1 (mean value of 2.4 g with a standard deviation of 0.2) and effective solubility of 0.13 g CA / kg CO_2 at 30 MPa and 313 K was estimated. The solubility of CA in $SCCO_2$ with ethanol cosolvent has been reported previously [23]; extrapolation of these data to zero ethanol content provides a solubility of 0.04 g CA / kg CO_2, that is around three times lower than the value estimated from the extractions assays carried out in this work.

As expected, the EC_{50} values of S1 fractions are significantly lower than the EC_{50} values of S2 fractions, indicating that a selective precipitation of the antioxidant compounds was achieved in S1 separator. Nevertheless, no significant improvement of the antioxidant activity of the fractions collected in S1 is observed when EP1 is larger than 1 hour. Figure 3 shows the amount of CA recovered in the different samples obtained.

In accordance with the lower EC_{50} values obtained for the material recovered in S1 separator, it was obtained that carnosic acid is more favorable concentrated in S1 extracts (9 - 20 % wt) than in S2 fractions (2 - 8 % wt). Furthermore, the EC_{50} values of the fractions collected can be correlated with the carnosic acid content (see Figure 3) as was previously referred in the literature [14, 19].

As a result, it can be established that on-line fractionation is appropriate just during the first hour of extraction, since for EP1 $\geq$ 1 the antioxidant activity of the fraction collected in S1 separator remains almost constant ($EC_{50} \approx 16$). That is, once the essential oil compounds are removed from the plant matrix, the composition of the extracted material remains almost constant during the rest of the extraction and thus, the similar concentration of antioxidants provide similar antioxidant activity. Furthermore,

the yield of the fraction collected in separator S1 of Ext. 2 is 4.7 times higher, but with similar antioxidant activity, than the fraction obtained in Ext. 6 in which fractionation was accomplished during the whole extraction time.

Conclusions

The fractionation of rosemary supercritical extracts to get a product with high antioxidant activity was studied. Extractions were carried out at 313 K and 30 MPa, but varying the fractionation procedure using a two-stage depressurization system. The fractionation scheme proposed comprises two different periods of time. During the first period exhaustion of the essential oil from plant matrix was achieved, whilst the antioxidant compound were precipitated in the first separator cell. In the second period the extract was precipitated in the first separator and mixed with the material recovered during the first period. Using this procedure, a fraction was produced which contains a 2-fold increase of carnosic acid content in comparison with the product obtained when no fractionation is applied. Further, the yield of the antioxidant fraction produced is almost double the one obtained when fractionation is applied during the whole extraction time.

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Table 1. Supercritical CO_2 extraction of rosemary leaves at 313 K and 30 MPa: fractionation period (EP1) and extraction yields obtained. Total extraction time = 5 h. Extraction yield is referred to the plant raw material loaded to the extraction cell.

Ext.	EP1 (hours)	Extraction yield in separators (%)		Overall extraction yield (%)
		S1	S2	S1 + S2
1	0	4.35	-	4.35
2	1	2.80	1.42	4.22
3	2	1.62	2.28	3.90
4	3	1.31	2.77	4.09
5	4	1.11	2.90	4.01
6	5	0.60	3.75	4.34

Table 2. Volatile essential oil compounds identified by GC-MS in rosemary extracts.

retention time	component *i* identification	% Area										
		Ext 1	Ext 2		Ext 3		Ext 4		Ext 5		Ext 6	
		S1	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2
8.148	α-pinene	1.32	0.81	0.37	0.80	1.83	1.50	0.95	1.48	1.14	n.d.	1.00
13.083	limonene	1.33	1.34	0.84	1.20	1.25	1.70	1.59	1.11	1.14	n.d.	1.28
13.191	1,8 cineole	53.1	59.2	37.1	59.3	49.4	62.9	50.4	64.1	47.5	72.0	50.2
17.243	linalool	1.01	1.18	1.77	1.09	1.36	1.17	1.37	1.17	1.51	n.d.	1.34
19.645	camphor	19.2	19.8	25.8	20.6	22.6	20.1	22.2	21.7	22.4	28.0	21.4
20.933	borneol	6.79	4.26	8.69	4.97	6.14	2.95	5.96	2.48	6.78	n.d.	6.48
21.626	terpineol	1.72	0.94	2.10	1.00	1.48	0.84	1.54	0.97	1.66	n.d.	1.67
22.411	α-terpineol	6.29	4.40	8.84	4.47	6.30	3.46	6.06	2.60	6.91	n.d.	6.29
23.400	verbenone	3.35	3.64	5.81	3.10	3.96	2.78	4.02	2.74	4.33	n.d.	4.34
37.622	β-caryophyllene	3.39	1.91	6.29	2.20	4.05	1.93	4.22	1.34	4.40	n.d.	4.00
58.245	ferruginol	1.69	1.47	0.92	0.82	0.69	0.62	0.68	0.30	1.09	n.d.	0.99
Total absolute area x 10^{-6}		3.1	2.9	4.8	3.1	7.4	3.3	7.8	3.3	6.0	0.8	5.0

n.d.: non detected

Table 3. Concentration factors (r_i = chromatographic area in sample / chromatographic area of S1 Ext1 sample) obtained for the volatile essential oil compounds identified by GC-MS.

retention time	component *i* identification	Concentration factor, r_i									
		Ext 2		Ext 3		Ext 4		Ext 5		Ext 6	
		S1	S2	S1	S2	S1	S2	S1	S2	S1	S2
8.148	α-pinene	0.59	0.44	0.61	3.31	1.23	1.83	1.18	1.69	n.d.	1.21
13.083	limonene	0.97	0.98	0.92	2.26	1.39	3.04	0.89	1.67	n.d.	1.55
13.191	1,8 cineole	1.06	1.08	1.13	2.22	1.28	2.41	1.28	1.74	0.35	1.52
17.243	linalool	1.12	2.70	1.09	3.21	1.26	3.42	1.22	2.91	n.d.	2.12
19.645	camphor	0.99	2.08	1.09	2.82	1.13	2.94	1.20	2.28	0.38	1.80
20.933	borneol	0.60	1.98	0.74	2.16	0.47	2.23	0.39	1.94	n.d.	1.53
21.626	terpineol	0.52	1.88	0.58	2.05	0.52	2.27	0.60	1.88	n.d.	1.56
22.411	α-terpineol	0.67	2.17	0.72	2.39	0.59	2.44	0.44	2.14	n.d.	1.61
23.400	verbenone	1.04	2.69	0.94	2.83	0.90	3.05	0.86	2.52	n.d.	2.08
37.622	β-caryophyllene	0.54	2.87	0.66	2.86	0.62	3.16	0.42	2.53	n.d.	1.90
58.245	ferruginol	0.83	0.85	0.49	0.97	0.40	1.03	0.19	1.25	n.d.	0.94

n.d.: non detected

Table 4. Supercritical CO_2 extraction of rosemary leaves at 313 K and 30 MPa: carnosic acid content (% wt) and EC_{50} values of all fractions collected.

Ext.	EP1 (hours)	% wt carnosic acid		EC_{50} (μg/mL)	
		S1	S2	S1	S2
1	0	9.48	-	24.0	-
2	1	16.93	2.51	16.5	51.7
3	2	18.17	4.96	16.1	35.2
4	3	20.12	6.25	15.8	30.4
5	4	18.31	8.09	15.8	27.4
6	5	19.39	8.26	15.3	26.4

Figure captions

Figure 1. Scheme of the SFE experimental device.

Figure 2. Extraction yields obtained in the supercritical fractionation of rosemary extracts: (○) S1 separator; (△) S2 separator; (■) overall extraction yield.

Figure 3. Correlation between EC_{50} values and % wt carnosic acid (CA) of the rosemary fractions produced in Ext. 1 to 6: (○) S1 separator; (△) S2 separator.

$EC_{50} = 91.331 \cdot CA^{-0.596}$, $R^2 = 0.9968$.

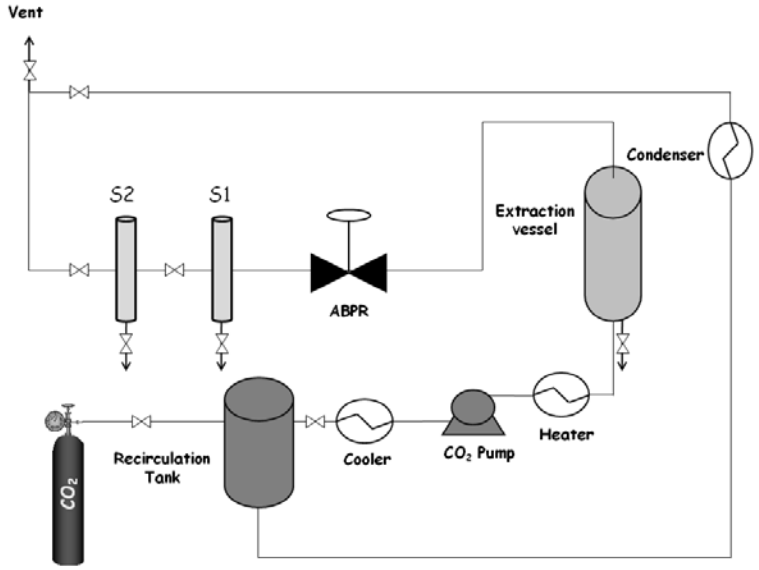
Vent
Condenser
S2
S1
Extraction vessel
ABPR
Heater
Recirculation Tank
Cooler
CO_2 Pump
CO_2

Figure 1

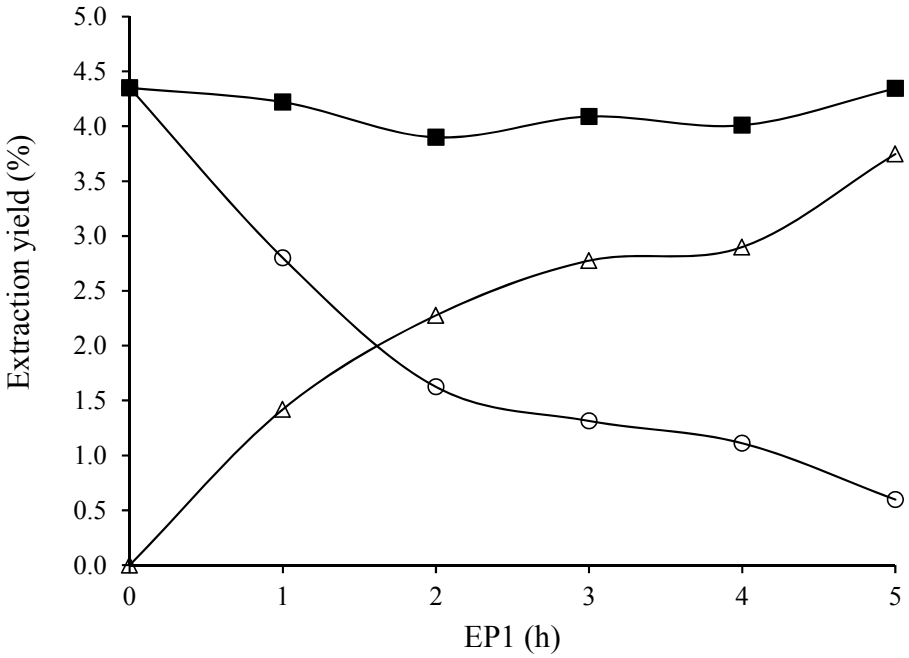

Figure 2

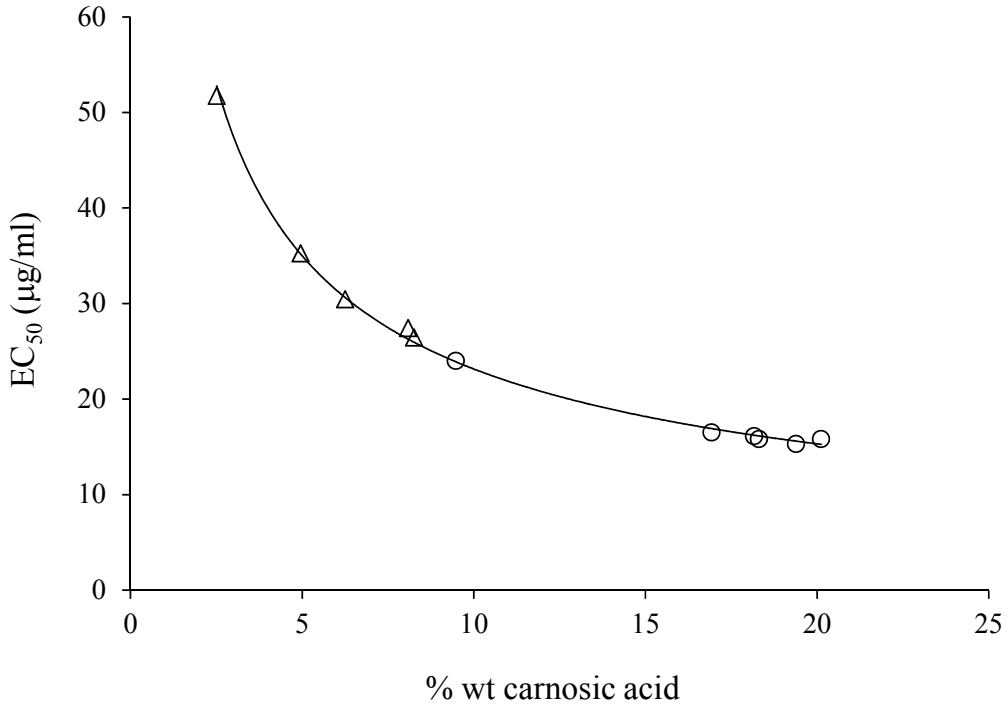

Figure 3

Short text for the table of contents

Supercritical CO_2 extraction of rosemary varying the fractionation time was studied to improve antioxidant activity. The best fractionation scheme comprised a first period to eliminate the essential oil from plant matrix, and a second period in which a product with high antioxidant activity was obtained. From this procedure resulted a product with 2-fold increase of carnosic acid content, high yield and antioxidant activity.